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Confocal microscopy of giant vesicles supports the absence of HIV-1 neutralizing 2F5 antibody reactivity to plasma membrane phospholipids

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ABSTRACT

The broadly neutralizing anti-HIV-1 2F5 monoclonal antibody recognizes a gp41 epitope proximal to the viral membrane. Potential phospholipid autoreactivity at cell surfaces has raised concerns about the use of this antibody for development of vaccines or immunotherapy. In this study, confocal microscopy of giant unilamellar vesicles (GUVs) was used to assess 2F5 reactivity with phospholipids assembled into bilayers with surface charge and curvature stress approximating those of the eukaryotic plasma membranes. Antibody partitioning into lipid bilayers required the specific recognition of membrane-inserted epitope, indicating that 2F5 was unable to directly react with GUV phospholipids, even under fluid phase segregation conditions. Our results thus support the feasibility of raising 2F5-like neutralizing responses through vaccination, and the medical safety of mAb infusions.

1. Introduction

The HIV-1 broadly neutralizing 2F5 monoclonal antibody (mAb2F5) recognizes the gp41 ELDKWA epitope within the "membrane-proximal external region" (MPER) domain of Env gp41 subunit (reviewed in [1–3]). This highly conserved gp41 domain is to date a major focus for HIV-1 vaccine development [1,2]. In addition, the mAb2F5 antibody is currently under clinical evaluation in passive immunization studies (reviewed in [4]). The fact that MPER may favorably insert into membrane interfaces, together with the presence of a long-hydrophobic CDR H3 loop, suggest that mAb2F5 might be adapted for recognition of its membrane-associated epitope [5–9].

Recognition in a membrane environment implies the establishment of intimate contacts between the antibody and the phospholipid bilayer. Accordingly, 2F5 was early described to bind directly, albeit with moderate affinity, to (mainly anionic) phospholipids, including the autoantigen cardiolipin, deposited on ELISA plates [6,10]. This phenomenon was interpreted as an evidence for autoreactivity mechanisms that would hamper anti-MPER neutralizing responses in vivo, and limit the potential use of anti-MPER mAb-s for passive immunotherapy [10,11]. Contrasting these observations, subsequent reassessments based on clinical ELISA failed to detect significant 2F5 reactivity with phospholipids [12,13]. It was further argued that anti-phospholipid reactivity was unlikely to evolve at the surface of the plasma membrane in healthy cells (see reference [14] for a discussion on this issue).

In this work, confocal microscopy of intact giant unilamellar vesicles (GUVs) was used for the first time to directly visualize mAb2F5 interactions with lipid bilayers bearing a curvature stress and surface charge comparable to those of the plasma membrane surrounding eukaryotic cells [15,16]. The plasma membrane bears an additional level of physicochemical complexity that was also taken into consideration in our study: the existence of laterally segregated lipid platforms or "rafts" [17,18]. Consistent with an antibody-membrane association process strictly dependent on

Abbreviations: Chol, cholesterol; DiD, 1,19-dioctadecyl-3,3,39,39-tetramethylindodicarbocyanine perchlorate; DiO, 1,1′-dioctadecyl-3,3′-oxacarbocyanine perchlorate; DOPC, dioleoyl-phosphatidylcholine; GUV, giant unilamellar vesicle; MLV, multilamellar vesicle; MPER, membrane-proximal external region; POPC, 1-palmitoyl-2-oleoylphosphatidylcholine; SLB, supported-lipid bilayers; SPM, sphingomyelin

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the specific binding to the membrane-inserted epitope, mAb2F5 partitioning into GUVs was only observed in the presence of epitope-peptide pre-bound to membrane. Quantitative flow-cytometry measurements further confirmed that mAb2F5 binding to vesicles was mediated by specific recognition of the membrane-inserted MPER epitope. Thus, our data support that mAb2F5 is not able to directly react with phospholipids accessible to the external leaflet of the plasma membrane, not even under fluid phase coexistence conditions.

2. Materials and methods

The NEQELLELDKWASLWNWFNITNWLWYIK (MPER-2F5) and NEQELLELAAWASLWNWFNITNWLWYIK (2F5-MPER(9,10)A) peptides were produced by solid-phase synthesis using Fmoc chemistry as C-terminal carboxamides and purified by HPLC at the Proteomics Unit of the University Pompeu-Fabra (Barcelona, Spain). Peptide stock solutions were prepared in dimethylsulfoxide (DMSO, spectroscopy grade) and the concentrations were determined using a Bicinchoninic Acid microassay (Pierce, Rockford, IL, USA). Neutralizing antibody expressing hybridomas were originally generated by combined PEG-electrofusion of peripheral blood mononuclear cells of HIV infected non-symptomatic patients [19]. The 2F5 mAb used in this study was subsequently produced in recombinant CHO cells after the subclass switch to IgG1 [20]. 1-Palmitoyl-2-oleoylphosphatidylcholine (POPC), dioleoyl-phosphatidylcholine (DOPC), sphingomyelin (SPM) and cholesterol (Chol) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). 5-Cholesten-3-one (cholestenone) was from Sigma–Aldrich (St. Louis, MO, USA). The *N*-(fluorescein-5-thiocarbamoyl)-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine (f-DHPE), 1,19-dioctadecyl-3,3,39,39-tetramethylindodicarbocyanine perchlorate (DiD), 1,1′-dioctadecyl-3,3′-oxacarbocyanine perchlorate (DiO), and Alexa Fluor 488 fluorescent probes and the Alexa Fluor 488 goat anti-human IgG were from Molecular Probes (Eugene, OR, USA). The PE-Cy5 mouse anti-human IgG was purchased from BD Biosciences.

GUVs of the desired lipid composition were prepared according to the electroformation method as described in [21]. Planar Supported-lipid Bilayers (SLBs) were prepared as described in [22]. Confocal fluorescence microscopy of GUVs and SLBs was performed on a commercial LSM510 system from Zeiss (Jena, Germany) with a laser scanning module and using multi-track mode [23]. Image processing and analyses were carried out with ImageJ (rsb.info.nih.gov/ij/).

Permeabilization measurements were done by adding the GUVs to a previously stirred PBS solution containing free Alexa Fluor 488 (molecular mass 720 Da) as a marker and the lytic 2F5-MPER pep-

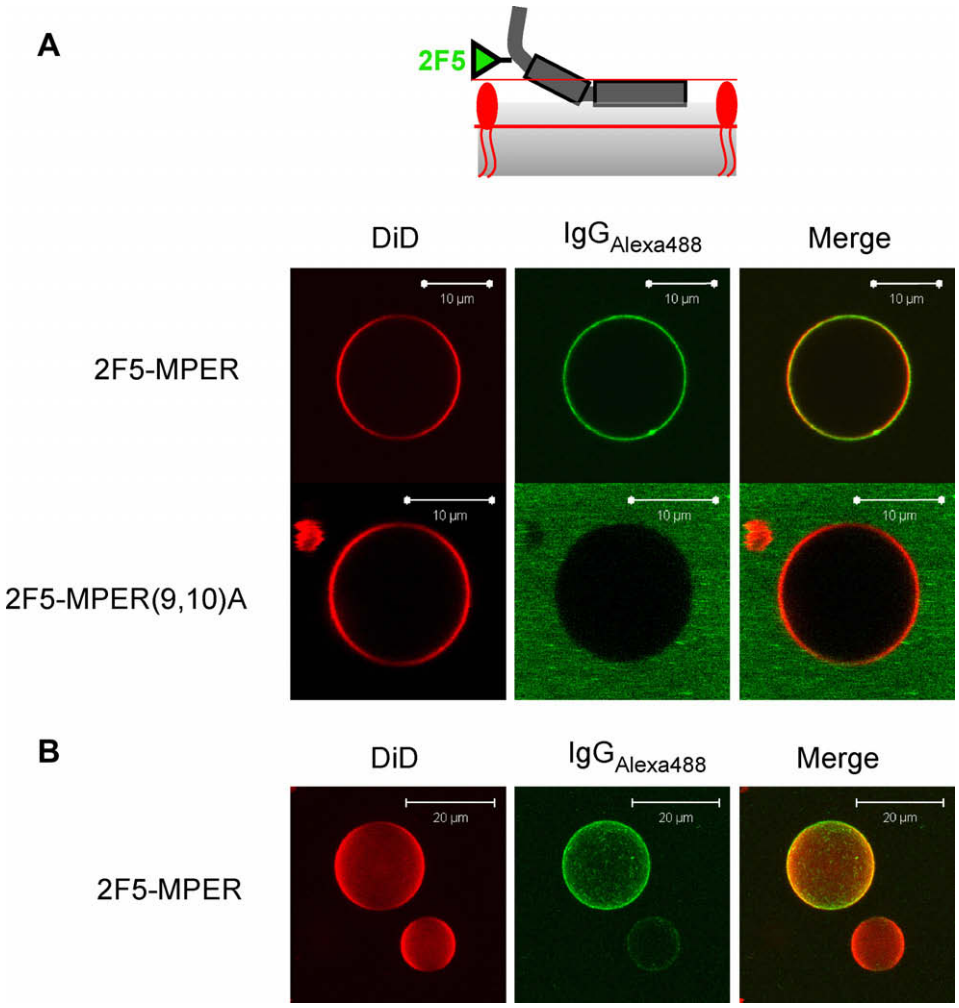

Fig. 1. Imaging of mAb2F5 binding to GUVs. (A) Confocal microscopy of GUVs (equatorial sections) electroformed from a mixture of POPC:Chol:DiD (4:1:0.01, mole ratio) containing 1% (mol:mol) 2F5-MPER or 2F5-MPER(9,10)A (top and bottom panels, respectively), and subsequently incubated with mAb2F5 (0.1 μM) for 1 h. 2F5 antibody was detected by incubating the mixture with Alexa Fluor 488 goat anti-human IgG (0.1 μM) for an additional hour. Lipid (red) and mAb2F5 (green) staining are shown in the left and center columns, respectively. The right column displays merging of both detection channels. The schematic diagram on top of the panels shows 2F5-MPER topology in relation to the membrane. (B) 3-D reconstruction of 2F5-MPER containing GUVs incubated with mAb2F5. Conditions otherwise as in the previous panel.

tide at the desired concentration. The sample was gently mixed to achieve a largely homogeneous distribution of vesicles, marker, and peptide. After 60 min the number of GUVs into which the marker had penetrated was counted versus the total number of vesicles in several regions of the sample.

Antibody-vesicle association was determined quantitatively using a BD FACScalibur Flow Cytometer (Becton Dickinson Immunocytometry Systems, Mountain View, CA) essentially as described in [7]. Measuring conditions were set for multilamellar vesicles (MLVs), a lipid concentration of 0.25 mM, and 0.01% fluorescent phospholipid probe (mole percent). In a typical experiment, fluorescently labeled MLVs doped with peptide epitopes (1:100 peptide:lipid molar ratio) were incubated for 10 minutes with increasing concentrations of the 2F5 mAb and subsequently, for 5 min with twice the concentration of anti-human immunoglobulin coupled to cyanine (IgG-PE-Cy5).

3. Results and discussion

3.1. Epitope binding-mediated mAb2F5 partitioning into GUV membranes

Most available data on mAb2F5-phospholipid reactivity have been obtained in ELISAs under conditions of uncontrolled phospholipid hydration, and/or using small vesicles composed of negatively charged lipids [6,10,24–26]. However, electrically neutral phospholipids are generally assumed to be the major components of the external leaflet of the plasma membrane [15]. Moreover, in the size scale of the phospholipid bilayer width (≈4–6 nm), the plasma membrane can be considered as a flat plane devoid of curvature stress. The absence of curvature stress, in combination with the molecular cohesion induced by the high levels of Chol [16], pose an effective restriction to the insertion of external agents into the lipid bilayer surrounding cells. Thus, giant vesicles with sizes comparable to those of cells and composed of POPC and Chol provide bona fide starting models to analyze mAb2F5 association with membranes emulating the external leaflet of the plasma membrane.

The confocal micrographs displayed in Fig. 1A correspond to equatorial sections of GUVs electroformed from a mixture of lipids and 2F5-MPER peptides and labeled with the lipophilic probe DiD (depicted in red). Control experiments using a fluorescently labeled peptide indicated that 2F5-MPER could be efficiently incorporated into GUV bilayers following this procedure (see Supplementary Fig. 1). GUVs containing peptide epitope were incubated with mAb2F5, which was subsequently imaged with a secondary antibody labeled with Alexa Fluor 488 (depicted in green). mAb2F5 could be detected in association with 2F5-MPER containing membranes following this procedure (Fig. 1A, top panels). The specificity of this binding process was tested using the 2F5-MPER(9,10)Ala peptide, in which Ala substituted for Asp9 and Lys10, two residues that are crucial for the neutralizing activity and binding of the 2F5 mAb [27]. In the 2F5-MPER(9,10)Ala-containing samples Alexastain appeared as a uniform bright background, against which the vesicles were seen as dark objects. This fluorescence pattern denoted that neither the labeled antibody, nor mAb2F5 associated with GUVs under these conditions (Fig. 1A, bottom panels).

The previous data clearly indicate that MAb2F5 partitioning into the POPC:Chol GUVs was not spontaneous, but mediated by the specific recognition of the 2F5 epitope sequence anchored to the membrane (see also Fig. 4 below). The 3-D reconstruction of the MAb2F5-labeled GUV-s showed a homogeneous distribution of the Alexa Fluor 488 label on the surface of the vesicle (Fig. 1B). Thus, in addition to the specificity of the process, epitope recognition in the lipid bilayer surface of GUVs did not result in the formation of discernable antibody aggregates.

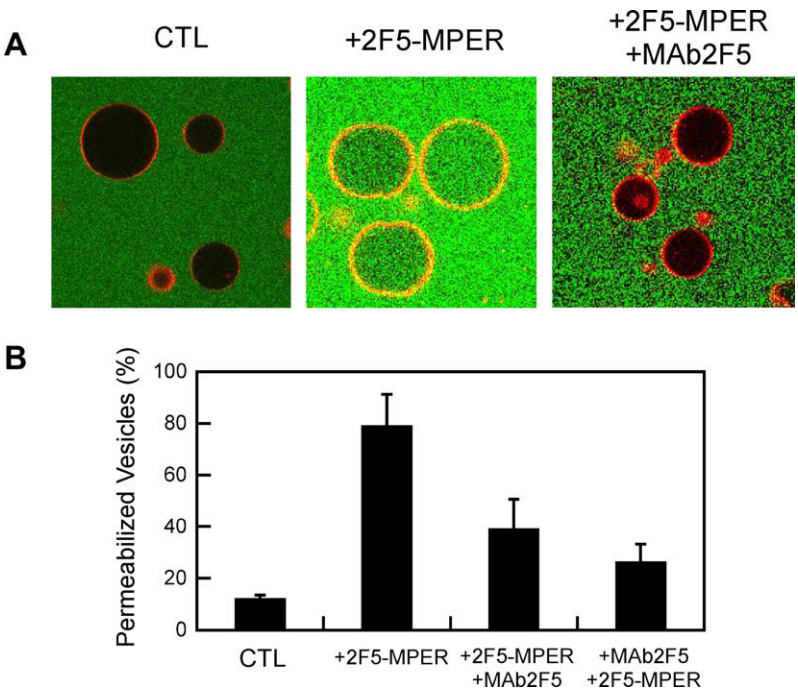

Fig. 2. Effect of mAb2F5 on 2F5-MPER permeabilizing activity measured in GUVs. (A) Confocal microscopy pictures of DiD-labeled POPC:Chol (4:1) GUVs after 2 h of incubation in a solution colored with soluble Alexa-488 (green) at 300 nM. CTL: untreated control vesicles showing unaltered permeability barrier. +2F5-MPER: vesicles incubated with 0.5 μM peptide that were permeabilized to Alexa-488. +2F5-MPER/+mAb2F5: vesicles were incubated with 0.5 μM peptide and, after 10 min, supplemented with 0.5 μM mAb2F5. The number of permeabilized vesicles was significantly reduced in these samples. (B) Quantification of the number of vesicles permeabilized in the previous samples. The +mAb2F5/+2F5-MPER column corresponds to a sample that contained 0.5 μM mAb2F5 in solution prior to the addition of GUVs and peptide. Average values of 2–4 fields counted ± S.D. are shown. All values were significantly lower ($0.001 < P < 0.01$) than the percentage of vesicles permeabilized by the peptide (+2F5-MPER column).

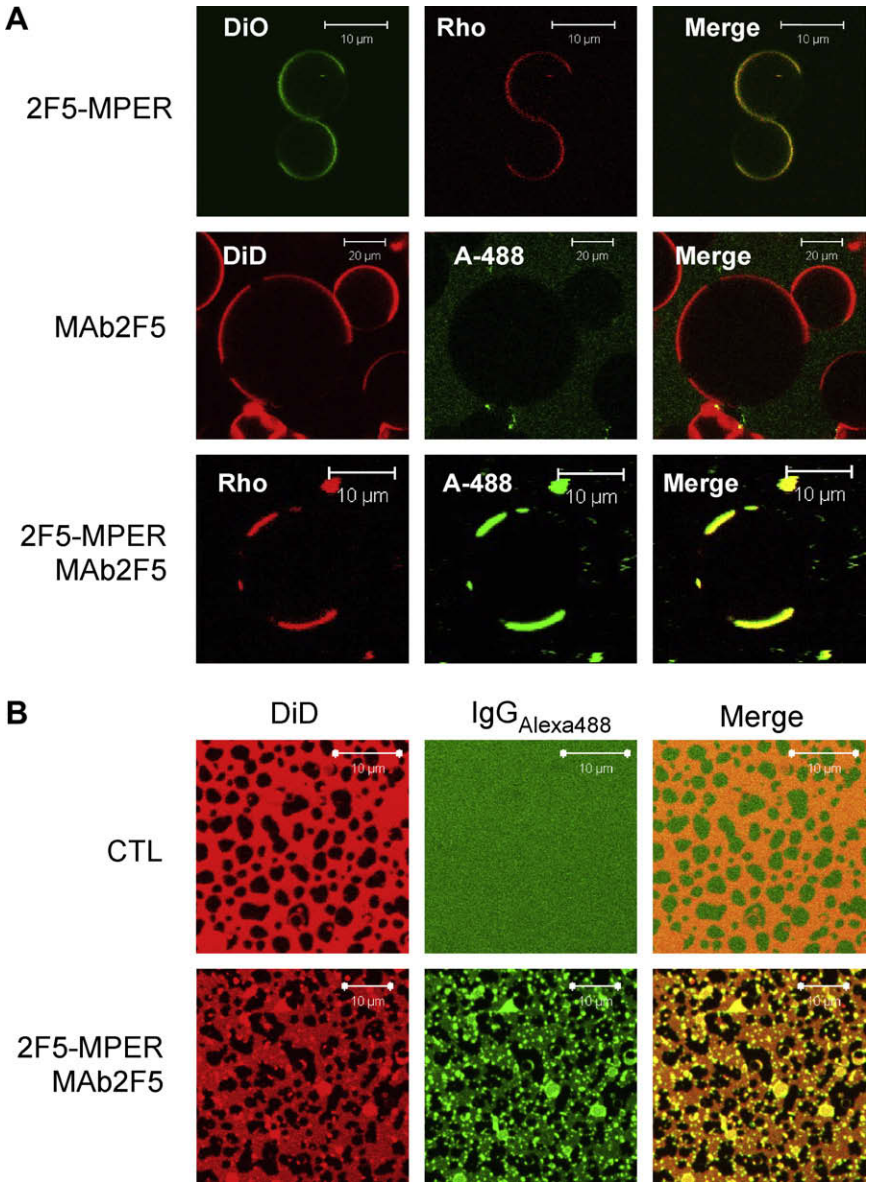

Fig. 3. Effect of fluid phase co-existence on mAb2F5 binding and peptide epitope recognition. (A) Top panels: confocal microscopy images of DiO-labeled DOPC:SPM:Chol (2:2:1) GUVs electroformed with 1% of rhodamine-labeled 2F5-MPER peptide. Lipophilic dye DiO (left) and rhodamine-labeled peptide (center) are depicted in green and red, respectively. DiO specifically partitions into Ld phase. Therefore, Lo domains are seen as dark areas in DOPC:SPM:Chol (2:2:1) samples. Peptide and lipid signals superimposed in both samples indicated that 2F5-MPER was excluded from the Lo domains (right). Middle panels: pictures of DiD-labeled DOPC:SPM:Chol (2:2:1) GUVs incubated with mAb2F5 (0.1 μM) for 1 h. DiD dye (red) specifically labels the Ld phase (left). The 2F5 antibody was detected with Alexa Fluor 488 goat anti-human IgG, but this dye remained soluble in solution (green background, center and right). Bottom panels: Images of DOPC:SPM:Chol (2:2:1) GUVs electroformed with 1% of rhodamine-labeled 2F5-MPER peptide and incubated with mAb2F5 (0.1 μM) for 1 h. The rhodamine label is depicted in red (left) and the 2F5 antibody was imaged with green Alexa Fluor 488 goat anti-human IgG (center). Both labels co-localize within the same domains (right). (B) 2F5 epitope recognition in supported-lipid bilayers. Confocal images of DiD-labeled DOPC:SPM:Chol (2:2:1) lipid bilayers deposited on mica supports. The untreated CTL samples (top panels) display the specific DiD labeling of Ld domains (depicted in red, left column). The green signal arising from Alexa Fluor 488 goat anti-human IgG is background noise and indicates no antibody binding (center column). Incubation with 2F5-MPER (0.1 μM) and mAb2F5 (0.1 μM) at intervals of 1 h (middle panels), gave rise to co-localization of DiD and Alexa Fluor 488 into Ld domains (right column). In these samples, brighter green spots could be observed against a more homogeneously labeled green background (center), which appeared as yellow spots in the merged image (right).

Evidence on epitope recognition at the GUV lipid bilayer surface was additionally obtained from the inhibitory effect exerted by mAb2F5 on MPER lytic activity (Fig. 2). The MPER domain has been proposed to be functional in HIV-1 fusion by perturbing the membrane following an activity pattern similar to that displayed by certain Trp-rich antimicrobial peptides [28]. 2F5-MPER added externally to the GUV suspension also incorporated efficiently into bilayers (Supplementary Fig. 1), and permeabilized these vesicles to a soluble dye (Supplementary Fig. 2). As shown in Fig. 2, this membrane permeabilization phenomenon could be significantly inhibited in GUVs by the addition of mAb2F5.

3.2. Fluid phase co-existence effect on mAb2F5 association with GUVs

Overall, the results displayed in Figs. 1 and 2 support the specific recognition and blocking of the membrane-buried 2F5 epitope under conditions that emulate the curvature stress and surface charge of the plasma membrane. They also rule out the existence of direct mAb association with the bare lipid bilayer under those conditions. However, in terms of modeling interactive properties of cell plasma membranes it is also necessary to account for fluid phase immiscibility [17,18]. It is generally assumed that certain proteins can selectively partition into lipid rafts [17], while insertion of others

has been postulated to occur at the lateral heterogeneities or defects that may exist within raft-domain boundaries [29]. The possibility existed that the mAb2F5 long-hydrophobic CDR H3 loop might intersperse between lipids at these membrane sites, thereby promoting antibody partitioning into bilayers under conditions of fluid phase co-existence. Therefore, in the next set of experiments we sought to analyze the effect of Lo (liquid-ordered) + Ld (liquid-disordered) lipid phase co-existence on antibody partitioning and recognition of the membrane-inserted epitope (Fig. 3).

GUVs composed of ternary mixtures of unsaturated PC, SPM and Chol mimic the chemical composition and the properties of the lipid rafts found in the plasma membrane [23,30]. Accordingly, GUVs composed of DOPC:SPM:Chol (2:2:1, mole ratio) show lipid phase separation (Fig. 3A). Vesicles in the top panels of Fig. 3A display Ld phase labeled with DiO (green), and mostly unlabeled Lo domains. Rhodamine-labeled 2F5-MPER peptide was found to co-localize with DiO, indicating that the peptide was segregated into the disordered Ld domains. Confocal images displayed in the middle panels rule out the possibility that the mAb2F5 molecule might specifically associate with rafts and/or directly insert into domain boundaries. In these samples DiD dye was used to label Ld phase (depicted in red). The Alexa-488-labeled secondary antibody (green) remained in solution, and could not be observed in contact with the vesicles upon incubation with mAb2F5. In sharp contrast, mAb2F5 associated with vesicles containing the 2F5-MPER peptide (bottom panels). The use of rhodamine-labeled peptide allowed the co-localization of 2F5-MPER and mAb2F5 within the fluid disordered Lo domains. The 3-D projections of representative GUVs displayed in the Supplementary Fig. 3 provide further evidence for the absence of 2F5-MPER or mAb2F5 aggregation at the surface of GUVs. They also rule out preferential peptide and/or antibody localization at the boundaries between domains.

In conjunction, the data presented in Fig. 3A demonstrate that under conditions allowing fluid phase co-existence, 2F5 epitope recognition also mediates the recruitment of mAb2F5 molecules into the surface of GUVs. Liquid domain co-existence and interacting proteins can be also directly visualized by confocal microscopy of supported-lipid bilayers (SLBs) [31]. In supported DOPC:SPM:-Chol (2:2:1) bilayers, DiD was also excluded from the ordered domains, which were observed as dark areas (Fig. 3B, left-hand panels). Similarly to what it was observed in the GUV system, mAb2F5 associated with the disordered domains in the presence of 2F5-MPER peptide. However, in comparison to the vesicular suspensions, in this system certain degree of mAb2F5 aggregation could be evidenced from the existence of spots with more intense green fluorescence. Given the fact that similar aggregates were not observed in association with GUVs, we infer that the formation of these aggregates is inherent to the SLB experimental conditions or sample preparation.

3.3. mAb2F5 association with lipid bilayers determined by flow-cytometry

Association of mAb2F5 with unstressed lipid bilayers was quantitatively assessed by flow-cytometry of MLVs (Fig. 4). For the case of 2F5-MPER-containing POPC:Chol vesicles, an increase in antibody fluorescence was apparent after incubation with a mAb2F5 dose of 20 μg/ml (green line in panel A, left). By comparison, for DOPC:SPM:Chol (2:2:1) vesicles containing 2F5-MPER peptide (B, left-panel), a distinct population of labeled vesicles was clearly discernible at a lower antibody concentration of 5 μg/ml (blue line), which increased in fluorescence intensity at 10 μg/ml antibody (red line). The negative control samples of vesicles containing 2F5-MPER(9, 10)A peptide (A, B, right panels) did not show a significant fluorescence increase even upon incubation with 20 μg/ml antibody.

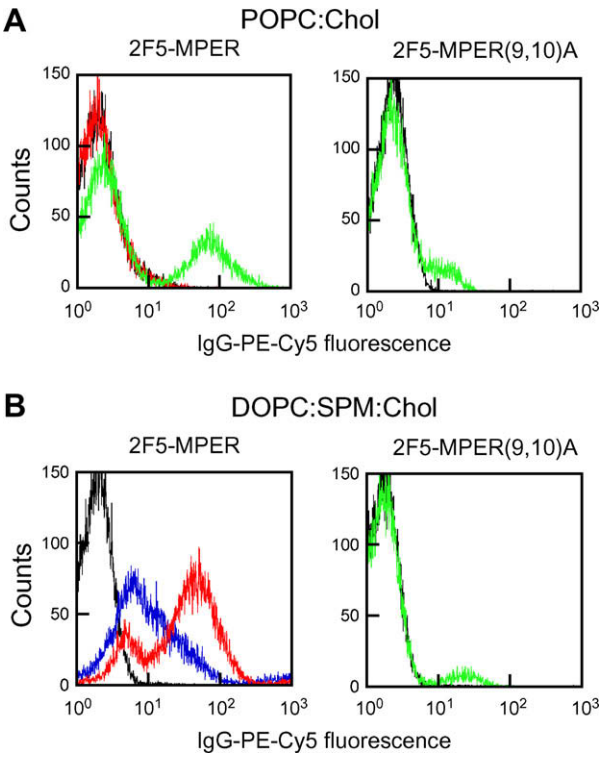

Fig. 4. 2F5 mAb association with 2F5-MPER-containing vesicles as determined by flow-cytometry. POPC:Chol (4:1) and DOPC:SPM:Chol (2:2:1) MLVs (panels A and B, respectively) were prepared by co-dispersing 2F5-MPER (left panels) or 2F5-MPER(9, 10)A (right panels) with lipids (1:100 peptide-to-lipid molar ratio). Vesicles (0.25 mM lipid) were incubated with 0 (black), 5 (blue), 10 (red) or 20 (green) μg/ml of mAb2F5.

Thus, the flow-cytometry results confirmed that antibody partitioning into both types of membranes was dependent on the specific recognition of the epitope sequence. Interestingly, they also revealed that the amount of mAb2F5 specifically bound to membrane-inserted epitope increased in the vesicles displaying fluid phase co-existence. The preceding confocal microscopy analysis was consistent with 2F5 epitope recognition occurring within Ld domains under phase co-existence. The confinement of 2F5-MPER into Ld domains results in the local increase of the peptide epitope surface concentration. Consequently, we infer that the more efficient mAb binding observed in Lo phase-containing vesicles can be attributed not to specific interactions with Chol or sphingomyelin but, rather, to an increase in the mAb binding avidity.

Finally, we note that our data do not rule out unambiguously the existence of mAb2F5 polyreactivity towards individual lipid species not taken into consideration in our study. In particular, Matyas et al. [26] reported certain degree of mAb2F5 binding to pure monohexosylceramides deposited on ELISA plates. While the ELISA may not be the method of choice to determine the binding specificity of the mAb2F5, the absence of binding to these lipids in the same ELISA assay of 2G12, an antibody binding to a gp120 epitope, implies a grade of specific recognition. Brügger et al. [32] determined that approximately 600 monohexosylceramide molecules are indeed incorporated into the raft virion membrane (i.e. ≈0.004 mol%). Thus, we decided to assay direct recognition of these molecules in the context of the unstressed membranes used in our study. The flow-cytometry results displayed in the Supplementary Fig. 4 indicate that inclusion of galactosyl ceramide (Gal-CER) at the membrane load used to test 2F5-MPER recognition (i.e., 1 mol%, Fig. 4, left panels) did not promote mAb2F5 association with vesicles. We conclude that this molecule was not directly bound by mAb2F5 under those conditions.

4. Concluding remarks

mAb2F5 polyreactivity with phospholipids has been claimed to limit its use in vaccine development and immunotherapy (see reference [14] for a comprehensive discussion on this issue). However, the capacity of 2F5 for binding phospholipids and the functional relevance of that phenomenon is presently a matter of debate. From a structural point of view, the presence of the hydrophobic CDR H3 loop, in combination with positively charged paratope surfaces, are likely to promote mAb2F5 adsorption to phospholipids immobilized onto solid supports [6,10,26]. This non-specific mechanism would also operate at the surface of small vesicles that are subject to lateral pressure allowing easier access to the hydrocarbon core [24].

In contrast, our GUV confocal microscopy study demonstrates that direct association of mAb2F5 with membranes does not occur in the context of unstressed bilayers mimicking the conditions existing at the plasma membrane. The undetectable water-membrane partitioning observed in the GUV system therefore supports the absence of mAb2F5 reactivity to phospholipids at the cell surface. The antibody was only found in contact with GUVs bearing surface-bound 2F5-MPER peptide. This finding reinforces the notion that stable antibody insertion into lipid bilayers may take place after specific recognition of membrane embedded epitope sequence, but not before.

Also in line with that idea, flow-cytometry measurements revealed an enhancement of mAb2F5 binding to membrane-inserted epitope in vesicles displaying fluid phase co-existence, consistent with the increase in surface concentration of MPER peptide segregated into Ld domains. We speculate here that this topological restriction might favorably affect the efficiency as multivalent immunogens of liposomal formulations destined for vaccination.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2010.03.021.

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